Electrophoretic Studies on Proteins In the Egg and Hemolymph of the Gypsy Moth With Reference to Isoenzymes

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Abstract: Egg proteins and hemolymph proteins of larval, pupal and adult forms of the gypsy moth (*Lymantria dispar*, Linnaeus) were analyzed electrophoretically. Six isoenzymes: polyphenol oxidase, malic acid dehydrogenase, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, alpha-glycerophosphate dehydrogenase, and lactic acid dehydrogenase were followed electrophoretically in the metamorphic forms of the gypsy moth. Changes in isoenzyme concentration were observed during development of the insect, and in some cases these differences were sex associated.

INTRODUCTION

The gypsy moth, *Lymantria dispar*, Linnaeus, is the major forest insect of the Northeastern United States. According to U.S. Forest Service estimates, the gypsy moth in a single year is capable of defoliating one and one half million acres of forest shade trees. Its hosts include most species of hardwoods; the oaks, gray birch, and poplar being most highly favored (Baker, 1972).

In efforts to control the population of this pest, studies by the U.S. Dept. of Agriculture dealing with applied and basic research have been in progress. With respect to basic research, a study of the biochemical properties of the blood or hemolymph of the gypsy moth has been a subject of interest in this laboratory. The present study was concerned with the electrophoretic examination of proteins in the diapausing egg, and in the hemolymph of larval, pupal, and adult forms. In addition, some isoenzymes were analyzed electrophoretically throughout the metamorphic stages of the insect.

MATERIALS AND METHODS

Insects. Insects were reared on a modification (ODell and Rollinson, 1966) of a wheat germ diet (Vanderzant et al., 1962) from egg masses collected in the Northeastern United States. Gypsy moth eggs contain a diapausing larva (Lees, 1955) which requires overwintering prior to hatch. A male larva has five instars while the female larva goes through an additional increase in size to form a 6th instar. The female pupa and female adult moth are larger in

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size than the male counterparts. In the laboratory the period from egg to adult lasts about seven weeks.

Samples. In the egg, larval, and pupal stages, pooling of samples was practiced in order to avoid variability in electrophoretic patterns noted in individual insects (Van der Geest and Borgsteede, 1969). Other reasons for pooling or non-pooling of samples, as for adults, are given under the stage described. Larval and pupal samples were obtained from the midpoint of each stage (Magnoler, 1970) in order to be as consistent as possible with respect to the time of development (Greene and Dahlman, 1973).

Egg Fraction. Field collected egg masses had been stored at 4 C for several months during the diapause period (Lees, 1955). Eggs ready to hatch were utilized. The egg masses were dehaired mechanically (Cosenza et al., 1963) and surfaced sterilized (ODell and Rollinson, 1966). To obtain a sample of sufficient protein concentration, 40 eggs were required. Eggs were homogenized in 0.4 ml phosphate buffer (0.01 M, pH 7) and centrifuged at 1600 *g* for 5 minutes to obtain the supernatant which was analyzed.

Larval Hemolymph. Larvae were bled by severing a proleg and hemolymph obtained from members of the same instar was pooled.

Pupal Hemolymph. Pupae were bled by piercing the integument at the junction of the wing and body (Loughton and West, 1965), and pooling was necessary to obtain sufficient volume. To observe whether sex-associated differences were present, hemolymph from male and female forms were obtained separately.

Adult Hemolymph. Adults were decapitated and the hemolymph collected at the exposed neck region (Laufer, 1960). To observe whether sex-associated differences were present, samples from each sex were obtained separately. Pooling of hemolymph was not feasible because of a lack of synchronization in the emergence of the adults. Since adults of certain insects lose blood volume readily (Gere, 1964), and to avoid changes upon storage, individual samples were run as soon as possible after emergence of the adults.

Electrophoresis. Samples of 10 μl were run by anodic polyacrylamide gel electrophoresis (PAGE) (Davis, 1964) with the following modifications: the spacer gel was excluded (Lynsenko, 1972); the system was run at 2.5 ma/tube (Wang and Patton, 1968) at 4 C in precooled buffer. To allow for better current conduction, the quantity of Tris was increased in the running gel from 36.3 g to 48.5 g, and in the sample gel from 5.98 g to 9.6 g (Personal Communication, E-C Apparatus Corp., 3831 Tyrone Blvd., N Street, St. Petersburg, Florida, USA). No melanization of hemolymph was observed if the samples were applied immediately to the gels and electrophoresed.

Proteins. After electrophoresis sample gels were immersed in naphthalene black (1% in 7% acetic acid) for 30 minutes at room temperature. The gels were destained in 7% acetic acid and were stored in this reagent.

Isoenzymes. The isoenzymes analyzed were the following: polyphenol oxidase (PPO), malic acid dehydrogenase (MDH), alcohol dehydrogenase (ADH), alpha glycerophosphate dehydrogenase (α-GPD), glucose-6-phosphate dehydrogenase (G-6-PD), and lactic acid dehydrogenase (LDH). The staining procedures were those of Shaw (1968) with the following exceptions PPO—60 mg of catechol was used as a substrate, in 100 ml of 0.01 M phosphate buffer, pH 6.8. The temperature of incubation for the staining solutions was 37 C. Samples were incubated until bands were fully developed, usually overnight (Grell, 1967). Sample gels were stained for protein in each run and employed as a standard for comparison.

PPO was tested in the egg, each larval instar, male and female pupae, and male and female adults. The remaining five isoenzymes were also determined in the metamorphic stages, except that in the larval stage observations were limited to the 5th instar.

RESULTS

Protein Banding. The patterns obtained for egg proteins and for hemolymph proteins of larval instars, pupae, and adults are shown in Fig. 1. The fastest migrating band was labeled number 1, and the sequence continues back in the direction of the origin (Brewer, 1970). The highest number of consistent bands observed, 13, were found in all stages except the egg fraction which gave a pattern of 8 bands. During development of the insect, bands 11, 7, and 6 showed little or no significant change. Observations noted for the other bands were as follows:

Band 13 was undetected in the egg. In the larval stage it reached its peak concentration in the 4th and 5th instars, and showed a marked decrease in the 6th instar. The band appeared to show no change in concentration in the male pupa, but in the female pupa, it was as concentrated as in the 4th and 5th instars. In the adult the band decreased in the female and was usually non-existent in the male.

In hemolymph of larval instars 1–6, and in the male pupa, one faint band, as shown in Fig. 1, was observed at various times between bands 13 and 12. On other occasions two faint bands were seen in this area.

Band 12 was present in all stages and was very predominant during development. The band increased in concentration from the egg up to the 5th instar, then decreased in the 6th instar. The band concentration showed no change in the pupal stages, but noticeably decreased in the male and female adult. As shown in Fig. 1 a faint band at times was noted between bands 12 and 11, in all stages except in the egg fraction and in hemolymph of larval instars 4 and 5.

Band 10 appeared in the egg fraction and increased in the larval stages with its maximum concentration in the 4th instar. The band decreased in the 5th instar and showed no further change.

Band 9 was present throughout the developmental stages. Its concentration fluctuated in all larval instars, but was significantly increased in the pupal forms. In the adult the band further increased and reached its peak activity in the female.

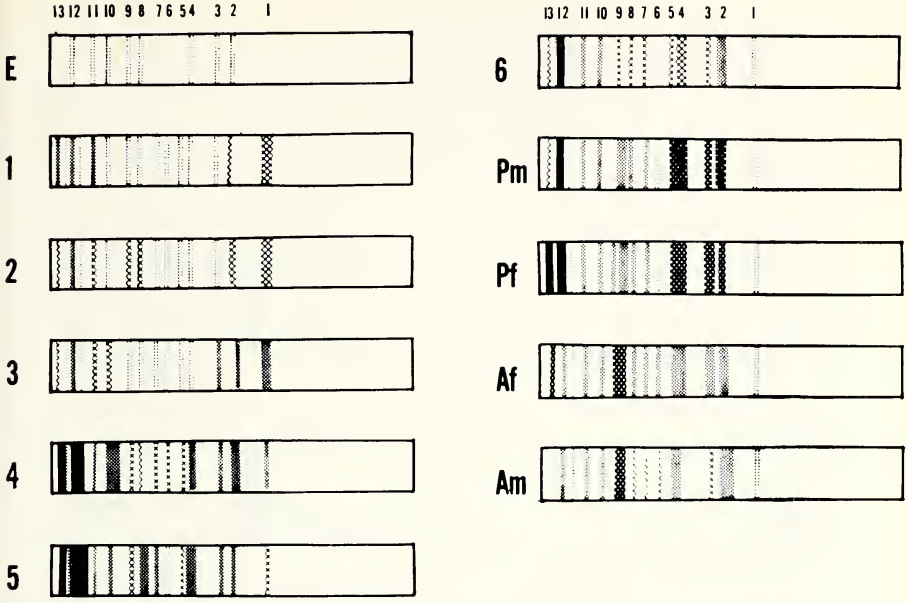

Fig. 1. Anodic Polyacrylamide Gel Electrophoresis (PAGE) of Egg and Hemolymph Proteins of the Gypsy Moth. The origin was at the extreme left of these patterns and those shown in Figs. 2 and 3. E = egg; nos. 1–6 = larval instars 1–6; Pm = male pupa; Pf = female pupa; Af = female adult; Am = male adult.

Band 8 was present in the egg fraction. Its maximum concentration was in the 5th larval instar with a noticeable decrease in the 6th instar. In the pupal forms and in the female adult the band increased slightly over that of the 6th instar, but then decreased in the male adult, equal in concentration to that in the 6th instar.

Band 5 was absent in the egg fraction. It appeared in the larval stages in low concentration. During the pupal and adult stages bands 5 and 4 increased in concentration and formed one band. In the pupal stage the area was much more intense than in the adult stage. The fused band was more concentrated in the male pupa than in the female pupa but was more concentrated in the female adult than in the male adult.

Band 4 appeared in the egg extract. In the larval stages it was fairly constant until the 4th instar where it began to increase up to the 5th instar, then decreased slightly in the 6th instar. In the pupal and adult forms, band 4 was fused with band 5, as described above.
Band 3 was present in the egg stage. It increased in concentration in the 3rd larval instar, remaining constant until it decreased slightly in the 6th instar. The band was increased markedly in the pupal stage over the concentration observed in the larval stages, and greater in the female pupa than in the male pupa. In the adult stage the band decreased and showed less concentration in the male than in the female.

Band 2 appeared in the egg stage. In the larval stage it increased steadily up to the 4th instar. The band increased further again in the pupal stage where it had a greater concentration in the male than in the female. It decreased in the adult stage showing a higher con-

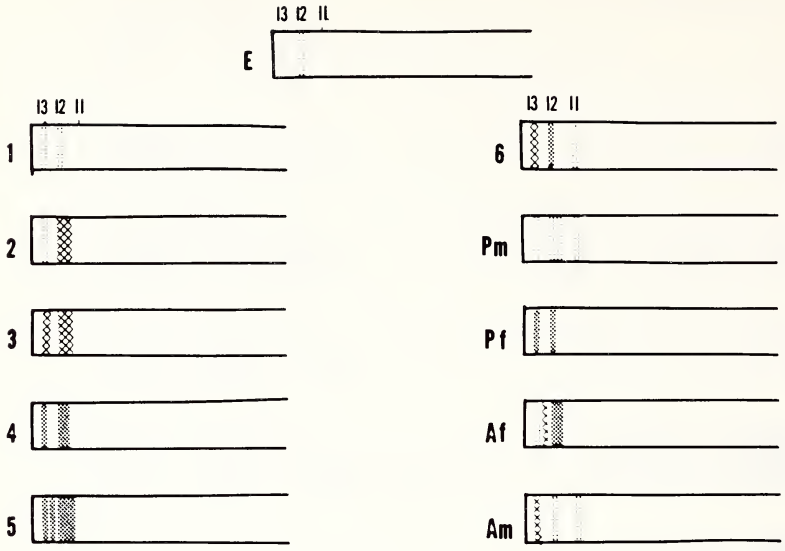

FIG. 2. Anodic PAGE of Polyphenol Oxidase in the Metamorphic Stages of the Gypsy Moth. Refer to Fig. 1 and the text.

centration in the male than in the female. It was noted that after extensive destaining, band 2 appeared to dissociate into 2 bands (not shown in Fig. 1).

Band 1 was absent in the egg. It appeared in the larval stage with maximum concentration in the 3rd instar. The band decreased progressively and remained at a low concentration throughout the rest of development.

Isoenzymes. PPO activity in the metamorphic stages of the gypsy moth was as follows (Fig. 2).

PPO in band 13. PPO was absent in this band in the egg fraction. In larval instars, PPO was present in the early stages, then began increasing at the 3rd instar, leveling off at the 4th and 5th instar, and decreasing in the 6th instar. The female pupa showed an increase in concentration over the 6th instar which was about equal in concentration to that observed for the 4th and 5th instars. There was a decrease in concentration in the male pupa over that noted in the 5th instar. In the adult forms the female decreased in concentration over that for the female pupa, while the male adult appeared to show a slight increase over the concentration noted for the male pupa.

PPO in the area between bands 13 and 12. Activity between bands 13 and 12 was detectable only in the 5th larval instar and the female adult; activity was higher in the 5th larval instar.

PPO in band 12. PPO activity was detectable in the egg fraction. Activity increased in the larval stages up to the 5th instar, and then decreased in the 6th instar. In the pupal stages the female activity was about equal to that of the 6th instar; the activity in the male pupa decreased. The female adult appeared to show a slight increase in activity over the female pupa while the male adult continued to show a decrease in concentration.

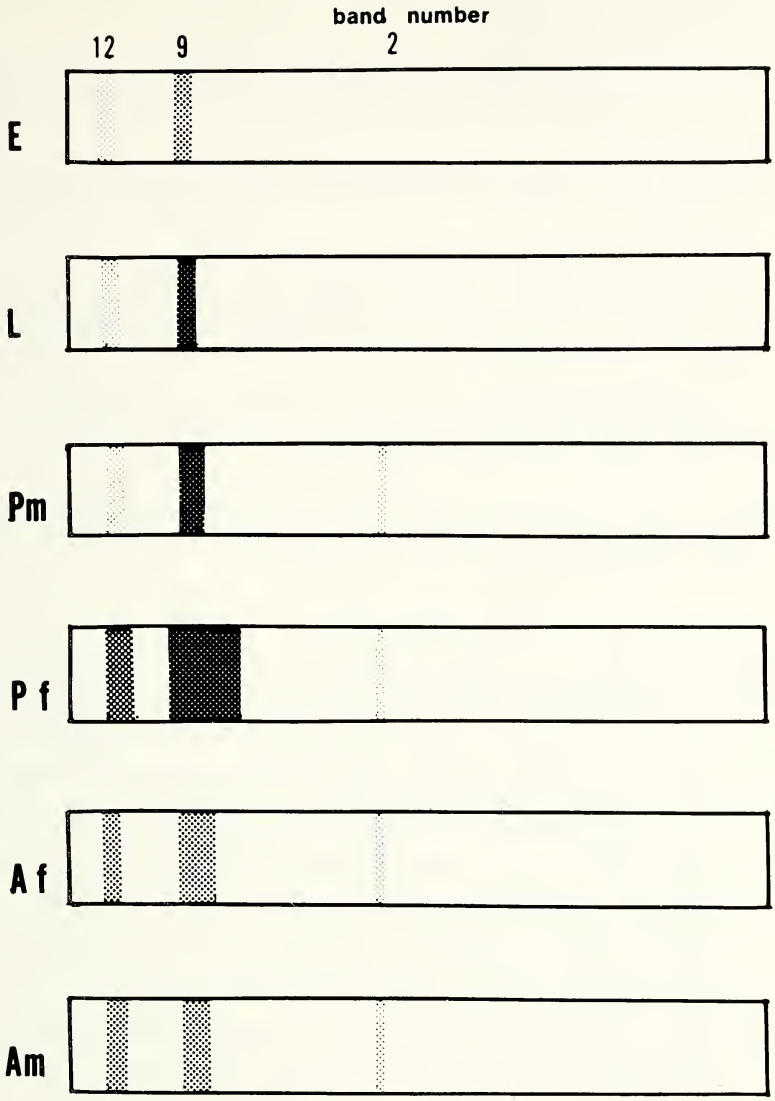

Fig. 3. Anodic PAGE of Malic Acid Dehydrogenase in various stages of the Gypsy Moth. Refer to Fig. 1 and the text. Activity in band 9 in the female pupa also extended into areas of bands 10 and 8.

PPO in band 11. PPO activity was not detected in the egg fraction. In the larval forms the activity was also generally absent in instars 1–5, but at times appeared in instars 3 and 4, (not shown in Fig. 3). The activity was present in the 6th instar but at a low level and was absent or very low (male pupa, male adult) in subsequent stages.

MDH. As shown in Fig. 3 the overall concentration was maximal in the pupal stages. The bands in the female pupa showed greater activity than in the male.

It was observed that ADH, α-GPD, G-6-PD, and LDH were all detected in samples of the egg fraction. However, in later stages, variabilities were noted in band number and intensity, and activity was expressed in terms of total staining of the bands. Additional observations were as follows:

ADH. The activity was maximal in the 5th larval instar, then decreased in succeeding stages with no significant differences noted between the male and female of the pupal and adult stages. Major bands were 12 and 11.

α-GPD. Activity was maximal in the 5th larval instar, decreased slightly in the pupal stage, and increased again in the adult stage, approximating the level of the 5th larval instar. There appeared to be no significant difference in activity between the sexes in the pupal and adult forms. A major band corresponding to protein bands 7, 6 and 5 (7–5) was observed.

G-6-PD was maximal in the 5th larval instar, then decreased with the remainder of insect development. The bands in the female pupa and female adult were slightly more concentrated in the male forms. Band 10 was a major band.

LDH was maximal in the 5th larval instar, decreasing in the pupal stage and further in the adult. There seemed to be no marked differences in intensity between the sexes in either the pupal or adult stage. Bands 13 and 12 were major bands.

DISCUSSION

Characteristic bands have been reported in electrophoretic patterns from insects. Van der Geest and Borgsteede (1969) noted an increase in concentration of "slow moving fractions" toward the end of larval life in *Pieris brassicae,* and a decrease in these "high molecular weight proteins" during the pupal stage. Hudson (1966) referred to a "common" blood protein, band 11, in the larval stage of the tomato hornworm. She believed it was similar to: (a) the main fraction in the hemolymph of *Galleria mellonella* (Denuce, 1958); (b) band 1 in the hemolymph of *cecropia* and *cynthia* larvae (Laufer, 1960); (c) the "common protein" noted by Whittaker and West (1962) in hemolymph from 18 insects; and (d) band 6 in the hemolymph and tissue of *Malacosoma americanum* and *Rothschildia orizaba* (Loughton and West, 1965).

Observations in the anodic PAGE system for gypsy moth center on predominant bands during the following stages: *larva*—13, 12; *pupa*—13 (female), 12, 9, 5–4 (combined), 3, 2; *adult*—9, 5–4 combined, 3 (female), 2.

Enzymes have been followed in various stages of insect development (Laufer, 1961; Chen and Levenbook, 1966; Knowles and Fristrom, 1967; Wright and Shaw, 1969).

PPO was maximal in the hemolymph of the 5th instar (Fig. 2). In insects PPO, in addition to its role in the tanning and hardening of the cuticle (Brunet, 1965), may be implicated in a defense function against invading microorganisms and parasites (Taylor, 1969).

ADH, LDH, and G-6-PD were maximal in hemolymph of the 5th instar,

decreasing proportionately in later stages. α-GPD did not show a general decrease in concentration in later stages from a maximal level in hemolymph of the 5th instar. The enzyme decreased slightly in the pupal stage but then increased in the adult, approximating the concentration in the 5th instar.

Unlike the other isoenzymes followed in the development of the gypsy moth, MDH was maximal in the female pupa (Fig. 3). The reduced activity in hemolymph of the male pupa was approximately equal to that observed in the hemolymph of the 5th instar.

In studies on the flight muscle of the African locust (*Locusta migratoria*), Bücher (1965) reported involvement of MDH, LDH, α-GPD, and G-6-PD. α-GPD has also been implicated in diapause metabolism (Gilmour, 1965).

Sex related differences have been cited in the literature with respect to electrophoretic patterns of insect material. Some investigators believed that these differences were in terms of protein levels (Stephen and Steinhauer, 1957; Hudson, 1966). By this parameter the present study demonstrated sex-associated differences for certain protein bands. *Band 13* was reduced in the male pupa in comparison to the female pupa. *Band 13*, present in low concentration in the female adult, was absent in the male adult. In the adult, *Bands 9 and 8* were more concentrated in the female. *Bands 5–4* (combined) were slightly higher in the male pupa, in comparison to the female pupa, but in the adult forms, the reverse was true. In the pupae, *Band 3* was slightly higher in the female, and in the adult forms, significantly higher in the female. *Band 2* was slightly higher in the male pupa and male adult in comparison to the female of these stages.

Sex-associated differences observed for the isoenzymes followed in the development of the gypsy moth were as follows: *PPO* was greater in hemolymph of the female pupa and female adult than in the male of these stages (Fig. 2). *MDH* was significantly higher in hemolymph of the female pupa in comparison to the male, and slightly higher in the female adult in comparison to the male (Fig. 3). G-6-PD was observed to be slightly more concentrated in hemolymph of the female pupa and female adult than in the male of these stages.

A 6th larval instar occurs in the female of the gypsy moth. Although protein banding and isoenzyme analyses did not reveal any unusual observations, undoubtedly, this stage of development would demonstrate important changes in other compounds, such as hormones.

The egg of the gypsy moth contains a diapausing larva, and very likely hemolymph proteins are present at this stage. The egg extract was analyzed not necessarily for unique proteins, but to demonstrate the presence of isoenzymes in insect eggs (Agrell, 1964).

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